Carbohydrate Research, 80 (1980) 147–161

STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K17

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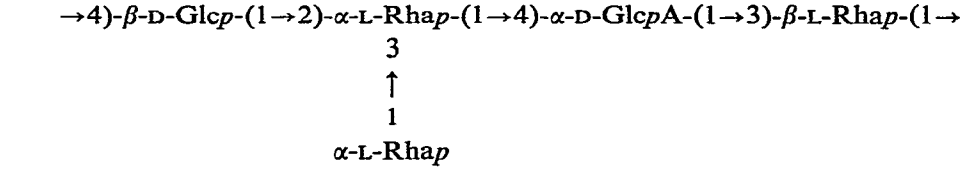
(Received August 18th, 1979; accepted for publication, October 24th, 1979)

ABSTRACT

The structure of the capsular polysaccharide from *Klebsiella* K17 is based on the pentasaccharide repeating-unit shown. The principal features of the structure were determined by standard methods, including ^{1}H- and ^{13}C-n.m.r. spectroscopy, which are shown to be complementary techniques. The position of the one β-L-rhamnopyranosyl residue was established by selective periodate oxidation of the terminal, lateral substituent, despite the presence of in-chain sugar-units possessing α-glycol systems.



INTRODUCTION

Approximately 80 strains of *Klebsiella* can be distinguished serologically due to the differences in their capsular polysaccharides. Nimmich[1] has shown that the chemogroup comprising D-glucuronic acid, D-glucose, and L-rhamnose includes the strains of *Klebsiella* K17, K23, K44, and K45. As part of our continuing study on the immunochemistry of this genus, we had previously determined the structure of the capsular polysaccharides from *Klebsiella* K23 (ref. 2) and K44 (ref. 3), and we now report our results on K17. Immunochemical cross-reactions predicted[4] that the polysaccharide from K17 would have L-rhamnosyl groups as nonreducing end-groups; this is fully confirmed by the present study.

RESULTS AND DISCUSSION

Composition and n.m.r. spectra. Klebsiella K17 bacteria were grown on an agar medium, and the capsular polysaccharide isolated was purified by one precipitation with Cetavlon. The product had a molecular weight of 9.4×10^5 and $[\alpha]_D$ +30°.

TABLE I

N.M.R. DATA FOR *Klebsiella* K17 CAPSULAR POLYSACCHARIDE, AND COMPOUNDS ISOLATED THEREFROM

Compound[a]	Δ[b]	^{1}*H-N.m.r. data*			^{13}*C-N.m.r. data*	
		$J_{1,2}$[c] *(Hz)*	*Integral, proton*	*Assignment*[d]	*p.p.m.*[e]	*Assignment*[f]
Glcβ(1→2)Rha-OH	5.36	1	0.5	α-Rha-OH	105.1	β-Glc
	4.84	< 1	0.5	β-Rha-OH	93.7	α,β-Rha-OH
	4.63	7	1	β-Glc	61.4	C-6 of Glc
1	1.28	6 ($J_{5,6}$)	3	CH_3 of Rha	17.6	CH_3 of Rha
GlcAα(1→3)Rha-OH	5.17	[b]	1.6	α-GlcA and α-Rha-OH	96.7	α-Rha-OH
					96.3	β-Rha-OH
	4.87	[b]	0.4	β-Rha-OH	94.4	α-GlcA
2	1.33	6 ($J_{5,6}$)	3	CH_3 of Rha	17.7	CH_3 of Rha
Glcβ(1→2)Rhaα(1→4)GlcAα(1→3)Rha-OH	5.17	[b]	1.5	α-GlcA and α-Rha-OH	105.0	β-Glc
					100.5	α-Rha
	5.11	2	1	α-Rha	96.6	α-Rha-OH
3	4.87	5	0.5	β-Rha-OH	92.2	β-Rha-OH

	4.57	7–8	1	β-Glc	94.4	α-GlcA
	1.31	6 ($J_{5,6}$)	6	CH_3 of Rha	61.4	C-6 of β-Glc
					17.3	CH_3 of Rha
$\xrightarrow{4}$Glc$\xrightarrow[\beta]{1\ 2}Rha\xrightarrow[\alpha]{1\ 4}$GlcA$\xrightarrow[\alpha]{1\ 3}Rha\xrightarrow{1}_{\beta}$	5.17	3	1	α-GlcA	104.8	β-Glc
	5.11	s	1	α-Rha	101.3	β-Rha
	4.87	s	1	β-Rha	100.3	α-Rha
P1a	4.56	7.5	1	β-Glc	96.1	α-GlcA
	1.34	6	3	Rha	61.4	C-6 of β-Glc
	1.27	6 ($J_{5,6}$)	3	Rha	17.5	CH_3 of Rha
$\xrightarrow{4}$Glc$\xrightarrow[\beta]{1\ 2}Rha\xrightarrow[\alpha]{1\ 4}$GlcA$\xrightarrow[\alpha]{1\ 3}Rha\xrightarrow{1}_{\beta}$ with Rha$\xrightarrow{1}_{\alpha}$ on Rha-3	5.15	3–4	1	α-GlcA	105.0	β-Glc
	5.04	[g]	2	α-Rha, α-Rha	103.2	α-Rha (terminal)
	4.85	s	1	β-Rha	101.3	β-Rha
	4.60	10	1	β-Glc and α-GlcA (H-5)	100.8	α-Rha
	4.51	7[h]	1		96.4	α-GlcA
	1.30	6 ($J_{5,6}$)	9	Rha	61.5	C-6 of β-Glc
K17 capsular polysaccharide					17.4	CH_3 of Rha

[a]For origin of compounds 1–3 and **P1a**, see text. [b]Chemical shift relative to internal acetone: δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.). [c]b = broad, unable to assign accurate coupling-constant; s = singlet. [d]For example, α-Rha = proton on C-1 of α-linked L-Rha residue. [e]Chemical shift in p.p.m. downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. downfield from D.S.S. [f]As for [d], but for anomeric, ^{13}C nuclei. [g]A pair of unresolved singlets. [h]Signal appears as a triplet.

Gas–liquid chromatographic analysis of an acid hydrolyzate of the polysaccharide, after reduction of the uronic acid[2,5], showed rhamnose and glucose to be present in the ratio of 3:2. Circular dichroism measurements on the alditol acetates demonstrated the rhamnose and the glucose to be of the L and the D configuration, respectively.

The ^{13}C-n.m.r. spectrum showed signals for five anomeric carbon atoms and indicated that C-6 of the D-glucose residue was unsubstituted. A signal at 175 p.p.m. was consistent with the presence of a uronic acid.

Initially, the ^{1}H-n.m.r. spectrum of K17 was difficult to interpret. The problem arose when six signals could be discerned in the anomeric region (δ 4.5–5.5); these were particularly clear in a spectrum obtained on a 250-MHz instrument. Assignment of the anomeric signals was finally achieved by examination of ^{1}H-n.m.r. spectra of poly- and oligo-saccharides derived from the capsular polysaccharide during the structural investigation (see Table I). Other studies in this laboratory[6] had demonstrated that it is possible for the n.m.r. signal of H-5 of D-glucuronic acid, especially when α-linked, to appear down-field, approaching the anomeric region (δ 4.5). Therefore, the "anomeric" signal at δ 4.51 has been attributed to H-5 of α-D-glucuronic acid. It is notable, also, that the signal at δ 4.60 has a larger coupling-constant (10 Hz) than that normally associated with a β-D-glucose component. The integral ratio of 5:9 for anomeric protons to rhamnose methyl protons demonstrates the presence of three rhamnose residues per repeating unit. The absence of pyruvate and acetate from K17 capsular polysaccharide was established by the ^{1}H-n.m.r. spectrum.

TABLE II

METHYLATION ANALYSES OF K17 CAPSULAR POLYSACCHARIDE AND DERIVED POLY- AND OLIGO-SACCHARIDES

Methylated sugars[a] *(as alditol acetates)*	T[b]	*Mole %*[c]					
		Ia[d]	*Ib*	*II*	*III*	*IV*	*V*
2,3,4-Rha	0.48	21.6	19.3	7.2[e]			
3,4-Rha	0.79		5.1	23.9	26.3		49.9
2,4-Rha	0.89	21.1	22.5	27.9	27.9	48.2	
2,3,4,6-Glc	1.00				30.6		50.1
4-Rha	1.25	21.8	15.8				
2,3,6-Glc	1.57	20.2	20.3	24.2			
2,3,4-Glc	1.61					51.8	
2,3-Glc	2.15	15.4	17.1	16.8	15.2		

[a]2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol, etc. [b]Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on column *B* (OV-17) programmed at 175° for 8 min and then 2°/min to 210°. [c]Values were corrected by use of effective carbon response-factors given by Albersheim *et al.*[30]. [d]Ia, original capsular polysaccharide; Ib, as for Ia, but partially degraded by ion-exchange resin; II, **P1**, **P1a** gave a similar result; III, acidic tetrasaccharide **3**; IV, aldobiouronic acid **2**; and V, neutral disaccharide **1**; see text for details. [e]A small peak of 2-*O*-acetyl-1,3,4-tri-*O*-methylerythritol also appears in the chromatogram.

Methylation analysis. Methylation[7,8] of the acidic polysaccharide, and subsequent reduction with sodium borohydride, hydrolysis, and derivatization as the alditol acetates, indicated that K17 is composed of a pentasaccharide repeating-unit (see Table II, column Ia). The presence of a mono-*O*-methylrhamnose residue is attributable to a branch point and that of a tri-*O*-methylrhamnose residue to the terminal sugar of a side chain. The 2,3-di-*O*-methylglucose is the only derivative that could have arisen from the D-glucuronic acid component. Positions of methylation in the derived alditol acetates were determined by mass spectrometry[9] (m.s.) and, in this investigation, individual stereoisomers were readily identified by their characteristic retention-times[9]; columns *B*, *C*, and *D* each gave satisfactory separations.

In a preliminary methylation-analysis, ~5% of 3,4-di-*O*-methylrhamnose was obtained (see Table II, column Ib); this was traced to hydrolysis of the side-chain rhamnose by prolonged contact with the cation-exchange resin used to convert the isolated polysaccharide into the acid form. When the time of contact was shortened, this adventitious hydrolysis did not occur. The formation of 3,4-di-*O*-methylrhamnose shows, however, that a side chain is attached at O-3 of the 2-linked rhamnose unit, as demonstrated more directly by subsequent, periodate-oxidation experiments.

Partial hydrolysis. Partial, acid hydrolysis of the native polysaccharide was followed by separation of the acidic and neutral fractions by ion-exchange chromatography. The neutral fraction contained monosaccharides, mainly rhamnose, and a disaccharide (**1**). Chromatographic separation of the acidic fraction yielded two acidic oligomers (**2** and **3**).

The ^{1}H- and ^{13}C-n.m.r. spectra (see Table I) of compound **1**, $[\alpha]_D$ +3.7°, contained one nonreducing, β-anomeric signal and two anomeric signals attributable to a reducing 6-deoxyhexose. N.m.r.-spectroscopic studies on this compound have been reported by King and Bishop[10] (^{1}H) and Colson and King[11] (^{13}C). Methylation, hydrolysis, and g.l.c.–m.s.[9] of the alditol acetates gave 2,3,4,6-tetra-*O*-methylglucose and 3,4-di-*O*-methylrhamnose (see Table II, column V). The structure of **1** is thus established as β-D-Glc*p*-(1→2)-L-Rha.

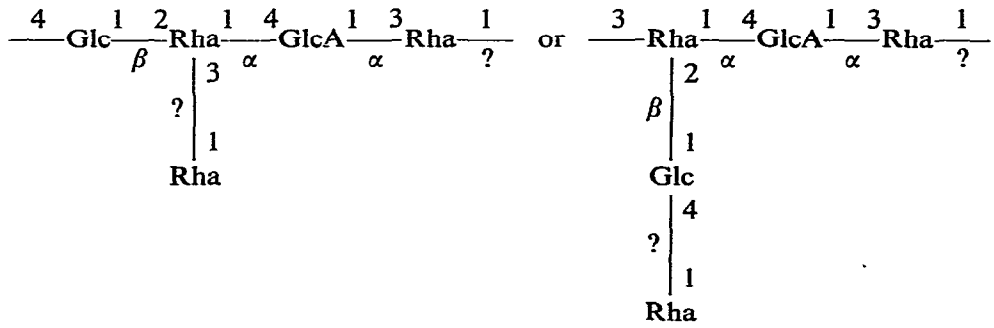
Compound **2**, $[\alpha]_D$ +111°, was shown by p.m.r. spectroscopy (see Table I) to give, in the anomeric region, one signal that was due to the combination of a nonreducing and a reducing α-glycosidic linkage and a second signal attributable to a reducing 6-deoxy-β-hexose; similar results were obtained from the ^{13}C-n.m.r. spectrum. Methylation of **2**, and subsequent reduction with lithium aluminum hydride, hydrolysis, and derivatization as the alditol acetates, gave products corresponding to 2,3,4-tri-*O*-methylglucose and 2,4-di-*O*-methylrhamnose, as analyzed by g.l.c.–m.s.[9] (see Table II, column IV). Compound **2**, the aldobiouronic acid, is thus established as having the structure α-D-Glc*p*A-(1→3)-L-Rha. This is the first occurrence, to date, of this aldobiouronic acid in a *Klebsiella* bacterial polysaccharide, although the β-linked analog has been found[12] in *Klebsiella* K47.

Compound **3**, $[\alpha]_D$ +38°, was shown by ^{1}H- and ^{13}C-n.m.r. spectroscopy to produce four signals in the anomeric region (see Table I). Methylation analysis (as described for compound **2**) yielded 2,3,4,6-tetra-*O*-methylglucose, 3,4-di-*O*-methyl-

rhamnose, 2,3-di-*O*-methylglucose, and 2,4-di-*O*-methylrhamnose (see Table II, column III). Having established the structures of **1** and **2**, it was possible to assign the structure of **3** as β-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-Glc*p*A-(1→3)-L-Rha.

Periodate oxidation. A sample of K17 polysaccharide was oxidized with periodate, the product reduced, and the product re-treated with periodate in order to ensure complete oxidation[13]. Analysis of the resulting polyol, after reduction of the uronic acid, indicated the presence of rhamnose and erythritol in the ratio of 1:1. These results showed that the glucose, the glucuronic acid, and one unit of rhamnose had contiguous hydroxyl groups, thus confirming the results of the methylation analysis.

Selective periodate oxidation. The preceding experiments provided certain useful, structural information, but left two important questions unanswered. (*1*) Is the side chain one or more units long? and (*2*) of the three rhamnose units, is the single β-rhamnosyl residue a lateral substituent or does it lie in the main chain? Knowing the structures of compounds **2** and **3**, the possible choices are restricted to the alternatives **4** and **5**, between which a distinction may be made on the basis of partial periodate-oxidation.



4 **5**

That there is an electrostatic repulsion between periodate ion and the uronate anion is well substantiated[14], but more recently, Painter and co-workers[15] demonstrated that, due to steric effects, there are marked differences in the rate of periodate oxidation. In the case of a structure such as **4**, the rate of oxidation of the 4-linked glucose unit is inhibited by steric hindrance, and that of the glucuronic acid by ionic repulsion, whereas, by contrast, the terminal rhamnose unit is freely accessible and should be oxidized rapidly. An attempt was, therefore, made to distinguish between structures **4** and **5** by applying the concepts enunciated by Painter and colleagues[15a]. A similar, selective periodate cleavage of pneumococcal S-14 polysaccharide has been reported by Ebisu and co-workers[15b].

In the first experiment, use of 0.05M sodium periodate caused the rapid consumption of 2.6 molecules of oxidant per repeating unit in 100 minutes. Following borohydride reduction, analysis by g.l.c. demonstrated the presence of erythritol, in addition to the glucose and rhamnose expected. Smith degradation[16] of a portion of

the oxidized and reduced polysaccharide gave a product (**P1**) that was polymeric, as judged by the fact that it did not dialyze, but the ^{13}C-n.m.r. spectrum showed signals attributable to erythritol, indicating, again, that some oxidation of the in-chain units had occurred.

A second experiment, using only a three-fold excess of 14mM sodium periodate, gave a consumption of 1.1 molecules of oxidant per repeating unit after 120 min. Smith degradation[16] gave an almost quantitative yield of a polymeric product (**P1a**) whose ^{1}H- and ^{13}C-n.m.r. spectra did not indicate the presence of any erythritol, and whose ^{1}H-n.m.r. spectrum, when compared to that of the original polysaccharide, showed the presence of one fewer α-linked sugar units (see Table I). The terminal rhamnose residue is thus α-linked, and it therefore follows, from **3**, that the β-L-rhamnopyranosyl unit is the one lying between the glucuronic acid and the glucose residues.

Samples of both **P1** and **P1a** were methylated, and analysis of the products showed (see Table II, column II) the formation of 3,4-di-*O*-methylrhamnose and the virtual elimination of the terminal rhamnose units. These data clearly substantiate structure **4**, but, in order to confirm this deduction, a β-elimination experiment was performed. It is likely that a structure such as **5** would also be amenable to selective oxidation, but methylation, following Smith degradation, would give 2,3,4,6-tetra-*O*-methylglucose (and not 3,4-di-*O*-methylrhamnose).

Uronic acid degradation. The permethylated polysaccharide was subjected to a base-catalyzed, uronic acid degradation[17] using dimethylsulfinyl anion. After neutralization, the degraded material was isolated, and then re-alkylated using ethyl iodide and silver oxide. Hydrolysis of the ethylated, degraded material, and analysis of the products by g.l.c.–m.s.[9] of their alditol acetates, gave the results shown in Table III,

TABLE III

G.L.C. ANALYSIS OF K17 POLYSACCHARIDE AFTER URONIC ACID DEGRADATION

Methylated sugars[a] *(as alditol acetates)*	T[b]		*Mole %*[c]		
	Column B[d] *(OV-17)*	*Column* E[e] *(HIEFF-1B)*	*I*[f]	*II*	*III*
2,3,4-Rha	0.54	0.42	16.6	19.3	17.6
3-Et-2,4-Rha[g]	0.57	0.37	19.9	19.0	22.5
2,4-Rha	1.00	1.00	17.6	16.6	11.7
4-Rha	1.41	1.55	14.1	11.9	16.8
2,3,6-Glc	1.78	1.94	31.8	33.2	31.4

[a]2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol, etc. [b]Retention time relative to 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-rhamnitol. [c]Values were corrected by using effective carbon response-factors given by Albersheim *et al.*[30]. [d]Program: 175° for 8 min, and then 2°/min to 210°. [e]Program: 160° for 8 min, then 2°/min to 200°. [f]I, uronic acid degradation of polysaccharide, isolation of products, and then ethylation, column *B*; II, same, column *E*; III, uronic acid degradation of polysaccharide with direct ethylation, column *B*; see text for details. [g]3-Et-2,4-Rha = 1,5-di-*O*-acetyl-3-*O*-ethyl-2,4-di-*O*-methyl-L-rhamnitol.

columns I and II. Better results were obtained (with a second sample) when the ethylation was conducted directly in the reaction flask, without prior isolation of the degraded material[18] (see Table III, column III).

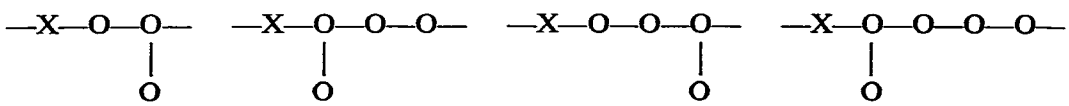
The presence of 3-*O*-ethyl-2,4-di-*O*-methylrhamnose indicated that rhamnose was the sugar attached to O-1 of the glucuronic acid, confirming the structure of the aldobiouronic acid as **2**. As some of the 2,4-di-*O*-methylrhamnose component was not ethylated, it may be assumed that all of the enol ethers formed during the elimination reaction were not cleaved, suggesting that mild, acid treatment[17] would have been appropriate in this case. Loss of the glucuronic acid residue involves cleavage of the main chain, and it was accompanied by some further degradation of exposed reducing-groups, as shown by lower relative-amounts of 4-*O*-methylrhamnose and 2,3,4-tri-*O*-methylrhamnose (see Table III). These data also support the concept that the branching rhamnose residue is attached glycosidically to O-4 of the glucuronic acid.

The results reported here demonstrate that the capsular polysaccharide from *Klebsiella* serotype K17 is composed of pentasaccharide repeating-units having the following structure.

$$\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow$$

$$\begin{array}{c} 3 \\ \uparrow \\ 1 \\ \alpha\text{-L-Rha}p \end{array}$$

Of the *Klebsiella* capsular polysaccharides whose structures have been reported to date, the pattern of having the uronic acid in-chain with a single substituent attached to a neutral sugar is common to several strains. Thus, *Klebsiella* K16 (ref. 19) and K54 (ref. 20) are of the "3 + 1" type, K7 (ref. 21) and K62 (ref. 5) of the "4 + 1" type, and K52 (ref. 22) represents the "5 + 1" arrangement. In this sense, the structure of K17 polysaccharide appears to resemble the pattern of K7 and K62, except that the position of the side chain relative to the uronic acid is different; the pattern of K17 is, therefore, unique. These patterns are illustrated schematically (where X = uronic acid, and O = neutral sugar, and such substituents as 1-carboxyethylidene or acetyl groups are ignored) as follows.



K16 (ref. 19), K54 (ref. 20) | K7 (ref. 21) K62 (ref. 5) | K17 | K52 (ref. 22)

The presence of a β-linked L-rhamnose residue in only one other *Klebsiella* capsular polysaccharide, that of serotype K32, has been reported[23]. However, in the present investigation, the absence of an isolated oligosaccharide with the β-L-rhamnose linkage intact, and the unusually high yield of compound **3**, lead to the conclusion

that the β-L-rhamnose linkage is more susceptible to acid hydrolysis than the comparable, main-chain, α-L-rhamnose linkage.

The n.m.r. spectra obtained in this study of K17 polysaccharide illustrate clearly that ^{1}H- and ^{13}C-n.m.r. should be considered as complementary techniques; each has certain advantages and disadvantages. It is generally assumed that, in ^{1}H-n.m.r. spectra of polysaccharides, anomeric protons will have chemical shifts in the range of δ 5.5–4.5, but the present investigation makes it plain that other ring protons may, under certain circumstances, have shifts that put them within this range. The anomeric configuration of the sugar, its pattern of substitution, and, for acidic polysaccharides, the pH of the sample solution are but three of the parameters that may influence chemical shifts of protons.

In ^{13}C-n.m.r. spectra, the chemical shifts of the anomeric carbon atoms of sugars having the *manno* configuration are so similar, regardless of whether they are α- or β-linked, that a definite assignment based on these shifts is problematical. In fact, the data on K17 polysaccharide show that a β-L-rhamnosyl signal lies between those for two α-L-rhamnosyl units. This conclusion was reached by comparing (Table I) the spectra for **3**, **P1a**, and the intact K17 polysaccharide. It is, of course, based on the assumption that no major alteration in chemical shift occurs when the structure is modified, *e.g.*, by removal of the side chain. On the other hand, ^{1}H-n.m.r. spectroscopy distinguishes clearly between rhamnopyranose in the α and β configurations (see Fig. 1).

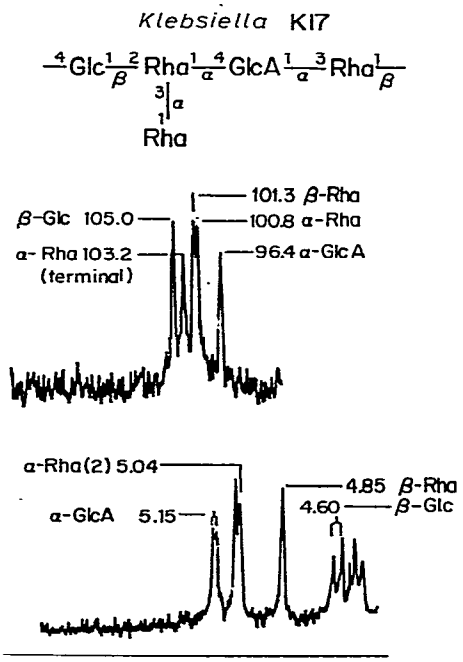


Fig. 1. N.m.r. spectra (anomeric region only) of *Klebsiella* K17 polysaccharide; upper, ^{13}C; lower, ^{1}H.

EXPERIMENTAL

General methods. — The instrumentation used has been described previously, and photocopies of the n.m.r. spectra recorded in Table I are available[3(a)]. Descending paper-chromatography was conducted by using Whatman No. 1 paper and the following solvent systems (v/v): (*1*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, and (*2*) 8:2:1 ethyl acetate–pyridine–water. Chromatograms were developed by using silver nitrate[24].

Analytical, g.l.c. separations were made in stainless-steel columns (1.8 m × 3 mm) with a carrier-gas flow-rate of 20 mL.min^{-1}, in conjunction with the following systems: (*A*) 3% of SP-2340 on Supelcoport (100–120 mesh); (*B*) 3% of OV-17, (*C*) 10% of OS-138, and (*D*) 5% of ECNSS-M, each on Gas Chrom Q (100–120 mesh); and (*E*) 3% of HIEFF-1B on Chromosorb W (80–120 mesh). Preparative g.l.c. was performed on column *F* (1.8 m × 6.3 mm) containing 5% of Silar 10C on Gas Chrom Q (100–120 mesh).

Gel-filtration chromatography was conducted on columns (1.5 m × 3 cm, or 1.0 m × 3 cm) of Bio-Gel P-2 (100–120 mesh). The columns were irrigated with 1000:10:4 water–pyridine–acetic acid at a flow-rate of ~10 mL.h^{-1}, and fractions (~2.0–2.5 mL) were collected.

Preparation and properties of Klebsiella K17 capsular polysaccharide. — A culture of *Klebsiella* K17 (2005/49) was obtained from Dr. I. Ørskov, Copenhagen, and was grown as previously described[2]. The cells were harvested after 3 d, diluted to 2 L with water containing 1% of phenol, and centrifuged, in batches, for 3 h at 34,000 r.p.m. in a Beckman model L3-50 ultracentrifuge with rotor type 35. The clear, supernatant liquors were decanted, combined (~1,300 mL), and poured into 10 L of ethanol. Crude polysaccharide, isolated by decantation and centrifugation, was dissolved in water (800 mL), and then precipitated with 10% Cetavlon solution. The precipitate was isolated by centrifugation, redissolved in 4M sodium chloride (800 mL), and reprecipitated by pouring into ethanol (8 L). The Cetavlon-purified polysaccharide was isolated by centrifugation, dissolved in water, and dialyzed for 2 d against running tap-water. Freeze-drying of this solution yielded 7.4 g of the sodium salt of the polysaccharide (~2.5 g per 2.5 L of medium), $[\alpha]_D$ +30° (*c* 0.57, water). Purity of the polysaccharide was checked by electrophoresis, using a 1% solution on cellulose acetate strips (Sepraphore III; 15 × 2.5 cm) in veronal buffer, pH 8.6 (LKB-Produkter AB, Stockholm 12, Sweden) at 300 V for 90 min, and then development in either Alcian Blue citrate-buffered ethanol (pH 4), or periodate–Schiff reagent. Homogeneity was also confirmed by gel chromatography, by courtesy of Dr. S. C. Churms, University of Cape Town, South Africa, and the molecular weight of K17 polysaccharide was determined to be 9.4 × 10^5.

The free-acid form of the polysaccharide was obtained by passing a solution of the sodium salt through a column of Amberlite IR-120 (H^+) resin, followed by freeze-drying.

Analysis of constituent sugars, and n.m.r. data. — Methanolysis of a sample

(30 mg) of *Klebsiella* K17 capsular polysaccharide with 3% methanolic hydrogen chloride, and subsequent treatment with sodium borohydride in anhydrous methanol reduced the uronic acid residues in the polysaccharide[2,5]. Total hydrolysis with 2M trifluoroacetic acid overnight at 95°, reduction of the liberated monosaccharides to alditols, and acetylation thereof, yielded L-rhamnitol pentaacetate and D-glucitol hexaacetate in the ratio of 8:5 (column *A*; programmed at 195° for 4 min, and then at 2°/min to 260°). Circular dichroism[25] of the rhamnitol pentaacetate showed $\Delta\varepsilon_{214}^{MeCN}$ −1.1, and the glucitol hexaacetate, $\Delta\varepsilon_{215}^{MeCN}$ +0.32, after collection of the alditol acetates by preparative g.l.c. (column *F*; programmed at 210°, and then at 4°/min to 250°).

Spectroscopic analyses were performed on the original K17 polysaccharide, but better spectra were obtained from material that had been partially depolymerized in order to lower the viscosity. This was achieved by mild hydrolysis in 0.4M trifluoroacetic acid for 15 min at 95°, and then several evaporations under diminished pressure with water, to eliminate the trifluoroacetic acid. The principal signals in the ^{1}H- and ^{13}C-n.m.r. spectra are recorded in Table I.

Methylation analysis of native polysaccharide. — A sample (285 mg) of K17 polysaccharide in the acid form was methylated by the methods of Hakomori[7,8] and of Purdie[26], and the product (283 mg) showed no hydroxyl absorption in the i.r. spectrum. A sample (35 mg) of this material was reduced overnight at room temperature[27] with sodium borohydride (100 mg) in 1:1 oxolane–ethanol. Hydrolysis of the methylated, reduced polysaccharide with 2M trifluoroacetic acid overnight at 95° was followed by reduction of the hydrolyzate with sodium borohydride, and acetylation of the resulting alditols. G.l.c. (column *C*, 190° isothermal; column *D*, 170° isothermal; column *D*, programmed at 180°, and then 2°/min to 220°) and g.l.c.–m.s. (column *B*) analysis of the methylated alditol acetates produced allowed making the assignments given in Table II, column Ia.

Methylation of a sample of K17 polysaccharide that had been de-ionized by stirring with Amberlite IR-120 (H^+) resin for two days gave the results shown in Table II, column Ib.

Partial, acid hydrolysis of polysaccharide K17. — Acidic polysaccharide (942 mg) was partially hydrolyzed in 0.1M trifluoroacetic acid for 3 d at 95° in an apparatus similar to that described by Galanos and co-workers[28]. After each 24-h period, the dialysis solution was changed, and the dialyzed material was isolated by removal of most of the water by concentration under diminished pressure and then freeze-drying the concentrate in a tared flask. As judged by the quantity of dialyzable material recovered, 80% was obtained after 24 h, and virtually 100% after 48 h. No residual polysaccharide remained in the dialysis sac. Paper chromatography (solvent *I*) of the dialyzed, hydrolyzed material showed the presence of several oligosaccharides, glucose, and a large proportion of rhamnose. The total, dialyzable material was made neutral with 0.1M NaOH, and applied to a column (30 × 1.5 cm) of Bio-Rad AG1-X2 (Cl^-) resin. The column was first eluted with water (1 L), yielding neutral mono- and oligo-saccharides, and then with 10% formic acid (100 mL), yielding the acidic

oligosaccharides (shown to be free from neutral components by paper chromatography in solvent *2*).

Gel chromatography (Bio-Gel P-2; 1.0 m × 3 cm) of the neutral components provided disaccharide **1** {9 mg; $[\alpha]_D$ +3.7° (*c* 0.82, water)}. The principal signals in the ^{1}H- and ^{13}C-n.m.r. spectra are reported in Table I.

Hakomori[7] methylation of **1** (6 mg) was performed in dimethyl sulfoxide (4 mL), using dimethylsulfinyl anion (2 mL, 4 h) and methyl iodide (1 mL, 1 h). The mixture was then transferred to a separatory funnel with water (15 mL), made neutral with 10% acetic acid (2 mL), and extracted with chloroform (3 × 10 mL). The extracts were combined, back-extracted with H_2O (4 × 10 mL), and evaporated to dryness under diminished pressure. Any residual solvent was removed by freeze-drying with water (15 mL) several times. Hydrolysis of the methylated disaccharide with trifluoroacetic acid (2M, 16 h, 95°), reduction, and acetylation yielded the alditol acetates from 3,4-di-*O*-methylrhamnose and 2,3,4,6-tetra-*O*-methylglucose in the ratio of 1 : 1 (g.l.c., column *B*). G.l.c.–m.s. confirmed the assignments of these components (see Table II, column V). Compound **1** is, therefore, 2-*O*-β-D-glucopyranosyl-L-rhamnose.

Gel chromatography (Bio-Gel; 1.5 × 3 cm) of the acidic components provided aldobiouronic acid **2** (47 mg) and acidic tetrasaccharide **3** (92 mg).

The aldobiouronic acid **2** (R_{Glc} 0.87, solvent *1*) had $[\alpha]_D$ +111° (*c* 1.12, water). The n.m.r.-spectral data are given in Table I. Hakomori[7] methylation of **2** (24 mg) yielded the permethylated aldobiouronic acid, which was then reduced with lithium aluminum hydride in refluxing oxolane. The neutral, methylated disaccharide was hydrolyzed, reduced, and acetylated, yielding equimolar amounts of 2,4-di-*O*-methylrhamnose and 2,3,4-tri-*O*-methylglucose as their alditol acetates (see Table II, column IV).

Compound **3** (R_{Glc} 0.48, solvent *1*) had $[\alpha]_D$ +38° (*c* 1.38, water); the n.m.r.-spectral data are given in Table I. Hakomori methylation of **3** (35 mg) gave the permethylated derivative, which was converted into the alditol acetates (as for compound **2**), yielding four components. G.l.c. and g.l.c.–m.s. (column *B*) identified these as being the alditol acetates of 3,4-di-*O*-methylrhamnose, 2,4-di-*O*-methylrhamnose, 2,3,4,6-tetra-*O*-methylglucose, and 2,3-di-*O*-methylglucose (see Table II, column III). The small proportion of 2,3-di-*O*-methylglucose is most likely to be due to an under-reduced product of the methyl ester after methylation of **3**.

Periodate oxidation of polysaccharide. — To a solution of the capsular polysaccharide (529 mg, in the sodium salt form) in water (100 mL) was added a solution (100 mL) that was 0.1M in sodium periodate and 0.4M in sodium perchlorate[14]. The solution was kept in the dark at 4°. After 120 h, ethylene glycol (10 mL) was added, and the solution was stirred for 1 h before dialysis for 3 d. Following reduction with sodium borohydride (1 g) for 24 h, addition of 10% acetic acid to decompose the excess of hydride (pH 6), and dialysis, the solution was freeze-dried, yielding 350 mg of polymeric material.

To ensure complete oxidation[13], the product from the first periodate oxidation

was dissolved in water (50 mL), an equal volume of the solution of sodium periodate (0.1M) and sodium perchlorate (0.4M) was added, and the mixture was kept in the dark for 94 h at 4°. Addition of ethylene glycol (5 mL), dialysis, and freeze-drying gave 324 mg of polyaldehyde. Further treatment with sodium borohydride (0.2 g), dialysis, and freeze-drying, yielded 303 mg of polyol.

Derivatives were prepared from a sample (15 mg) of the polyol by methanolysis with 3% methanolic hydrogen chloride, reduction with sodium borohydride in anhydrous methanol, hydrolysis, reduction, and acetylation. The product was analyzed by comparative g.l.c. (column *A*; programmed at 150° for 4 min, and then at 4°/min to 260°) with authentic standards. Only two compounds were present, identified as erythritol tetraacetate and rhamnitol pentaacetate, in the ratio of 1:1.

Selective, periodate oxidation of polysaccharide[15]. — To a solution of a sample (525 mg) of the polysaccharide in the sodium salt form in water (100 mL) was added 0.1M sodium periodate solution (100 mL). The reaction was allowed to proceed at 4° in the dark, and the periodate consumption was monitored by removing 1-mL aliquots which were analyzed by the Fleury–Lange method[29]. Periodate consumption reached a level of 2.6 molecules per repeating unit of *Klebsiella* K17 after only 1.7 h, whereupon the reaction was terminated with ethylene glycol (10 mL). Following dialysis, reduction with sodium borohydride, dialysis, and freeze-drying, 396 mg of polymeric material was isolated.

The isolated material (17 mg) was analyzed for sugar components in the usual way: methanolysis, reduction, hydrolysis, reduction, and acetylation. G.l.c. data (column *A*) showed that the peracetates of erythritol, rhamnitol, and glucitol were present in the ratios of 1:3.2:1.9. The volatile acetates of ethylene glycol and 1,2-propanediol were lost under diminished pressure during the processing.

The periodate-oxidized material (120 mg) was subjected to Smith degradation[16] using 0.5M trifluoroacetic acid for 17 h at room temperature. Following dialysis and freeze-drying (yield 59 mg), the resultant polymer (**P1**) gave signals in its ^{13}C-n.m.r. spectrum at 104.8, 101.2, 100.0, and 96.0 p.p.m. in the anomeric region. Signals were also observed at 63.4, 61.5, and 61.2 p.p.m., attributable to carbon atoms of primary alcohol groups (C-6 of a hexose and C-1 and C-4 of erythritol). Finally, two signals at 17.6 and 17.4 p.p.m. were assigned to CH_3 of two 6-deoxy sugars.

The oxidized and degraded polysaccharide **P1** was then methylated under Hakomori[7] conditions, followed by a Purdie[26] treatment (absence of hydroxyl absorption in the infrared spectrum). This methylated material was reduced overnight with lithium aluminum hydride in refluxing oxolane, hydrolyzed, reduced, and acetylated, with the results given in Table II, column II.

A second, selective, periodate-oxidation experiment was performed by dissolving K17 polysaccharide (289 mg, salt form) in water (50 mL), and adding a three-fold excess of sodium periodate (28 mM, 50 mL). Reaction conditions and analysis of periodate consumption were the same as already described. After 2 h, periodate consumption had reached 1.1 molecules per repeating unit, and the reaction was stopped by adding ethylene glycol; the polyol (283 mg) was isolated as already de-

scribed. The polyol (90 mg) was then subjected to Smith degradation, using 0.5M trifluoroacetic acid for 17 h at room temperature. After dialysis and freeze-drying, polysaccharide **P1a** was obtained in almost quantitative yield (76 mg); virtually no over-oxidation had taken place, as judged by the n.m.r. spectra. The main signals of the ^{1}H-n.m.r. spectrum (D_2O, 90°) of partially depolymerized **P1a** (0.4M trifluoroacetic acid, 20 min, 95°), and of the ^{13}C-n.m.r. spectrum, are given in Table I. Partially depolymerized **P1a** had $[\alpha]_D$ +44° (*c* 1.81, water).

Base-catalyzed degradation of methylated polysaccharide (uronic acid degradation)[17,18]. — A sample of carefully dried, methylated polysaccharide (99 mg) and *p*-toluenesulfonic acid (a trace) were sealed in a flask by means of a serum cap. With the aid of a syringe, dimethyl sulfoxide (19 mL) and 2,2-dimethoxypropane (1 mL) were added, and the mixture was stirred under nitrogen for several hours. Dimethylsulfinyl anion (10 mL) was then added by syringe, and the mixture was stirred for 18 h at room temperature. Following the slow addition of 50% acetic acid (~10 mL) to adjust the pH to 6, the solution was diluted with water (50 mL), and extracted with chloroform (3 × 25 mL). The extracts were combined, back-extracted with water (4 × 25 mL), evaporated to dryness under diminished pressure, and freeze-dried, to give a product that was ethylated with ethyl iodide and silver oxide. Analytical results are given in Table III, columns I and II.

A second, uronic acid-degradation experiment was performed on methylated K17 polysaccharide (36 mg) by the same procedure, with the exception that ethylation was conducted directly, without isolation of the degraded material[18]. That is, after reaction with dimethylsulfinyl anion for 18 h, ethyl iodide (3 mL) was added, dropwise, directly into the basic solution by using a syringe and external cooling. After 1 h, 50% acetic acid (10 mL) was added slowly to adjust the pH to 6. Extraction, back-extraction, and freeze-drying yielded the ethylated, degraded material. Hydrolysis, reduction, acetylation, and g.l.c. and g.l.c.–m.s. analyses showed that the same components were present as in the product of the first uronic acid degradation. However, a higher ratio of 3-*O*-ethyl-2,4-di-*O*-methylrhamnose to 2,4-di-*O*-methylrhamnose was indicated, even though not all of the 2,4-di-*O*-methylrhamnose had been ethylated (see Table III, column III).

ACKNOWLEDGMENTS

We thank Dr. I. Ørskov for a culture of *Klebsiella* K17 (2005/49), Dr. P. J. Salisbury for help with growing the bacteria, and Dr. A. A. Grey and A. Lee (Toronto) and Dr. M. Vignon (Grenoble) for recording some of the n.m.r. spectra. We are indebted to Merck Sharp and Dohme, and to NSERC, for financial support.

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